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Symposium on Replication of Viral Nucleic Acids

I. Formation and Properties of a Replicative Intermediate in the Biosynthesis of Viral Ribonucleic Acid[1]

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INTRODUCTION

Problem of Viral Ribonucleic Acid (RNA) Replication

Most deoxyribonucleic acid (DNA) viruses contain double-stranded DNA with the well-known duplex structure which replicates according to the mechanism first proposed by Watson and Crick (81). The replication of double-stranded viral DNA thus presents no particular problem beyond the basic problem of DNA synthesis, which has been studied for the most part with bacteria.

Some DNA viruses, such as ϕX174, contain single-stranded DNA (72), and during the course of infection intracellular double-stranded DNA is formed. This double-stranded DNA replicates extensively as a duplex, but it has not been clear whether the single-stranded DNA in the progeny virion is derived from the double-stranded *replicative form* (RF) by selection of one strand or by the synthesis of further single strands on the double-stranded template (73). Denhardt and Sinsheimer (17) recently presented a model for the replication of ϕX174 DNA, whereby the single-stranded parental DNA is incorporated into the RF duplex, which then replicates semiconservatively. The RF containing parental DNA may be responsible for the synthesis of all the single-stranded progeny DNA, whereas the other RF molecules probably do not replicate further but rather serve as templates for messenger RNA synthesis.

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Most RNA viruses contain single-stranded RNA (64, 66). The excellent studies of Gierer (29–31) showed that tobacco mosaic virus (TMV) RNA was a linear single-stranded polymer. Most later studies on viral RNA have not been as detailed, but in many cases the hydrodynamic properties of a particular RNA have been compared with those of TMV RNA. The data have suggested that the RNA of a virion exists as a single molecule (38). Analysis of the enzymatic inactivation of infectious RNA from enteroviruses also suggested that viral RNA is single-stranded (39). The hydrodynamic properties of bacteriophage R17 RNA are also compatible with those expected of a linear single-stranded polymer (53). Thus, there is a large amount of published data—some very convincing and some only suggestive—which indicate that most viral RNA is in the form of a single strand (64). Only two RNA viruses—reovirus and wound tumor virus—contain double-stranded RNA (32, 33).

There are two obvious alternatives for the chemical composition of viral RNA: it could be chemically homogeneous, or it could be a mixture of two species of polymers having defined linear sequences of bases, with one polymer being complementary to the other in base sequence. According to current belief, a given population of virions contains a homogeneous population of RNA molecules. This assumption is based chiefly on the base ratios of viral RNA, since generally the contents of adenine and guanine differ from those of uracil and cytosine, respectively (Table 1). The exceptions in Table 1 are reovirus and wound tumor virus, as expected. The adenine-uracil and guanine-cytosine ratios vary, but usually are not both equal to one for a

TABLE 1. *Base composition of the RNA from some RNA viruses*

Virus	Guanine	Cytosine	Adenine	Uracil	Reference
Bacteriophage					
R17	26.3*	24.9	23.1	25.7	53
Animal viruses					
Poliovirus (3 types)	24.0	22.0	28.5	25.4	65
Coxsackie A9	27.7	20.5	27.0	24.7	52
EMC	23.7	23.9	27.8	24.5	24
Influenza A(PR8)	20.1	24.0	23.1	32.8	63
Influenza B(LEE)	18.3	23.1	23.0	35.6	63
Rous sarcoma	20.5	30.4	29.9	13.2	62
Reovirus type 3	22.3	22.0	28.0	27.9	32
Plant viruses					
TYMV	17.2	38.1	22.6	22.1	51
Southern bean mosaic	26.0	23.0	25.8	25.3	20
TMV (normal)	25.3	18.5	29.8	26.3	46
Wound tumor	18.6	19.1	31.1	31.3	33

* Results expressed as moles per cent.

particular viral RNA. One cannot rule out the possibility of a constant fraction of complementary strands in a population of viral RNA. It would be extremely valuable to test further the presence or absence of complementary RNA in a viral population by attempted hybridization of a viral RNA with itself.

The data on single-strandedness and on base ratios suggest that there must be an asymmetrical step either in the synthesis of viral RNA or in the selection of viral RNA for assembly into virions. The asymmetrical step is probably that of synthesis, since intracellular viral RNA has the same base ratios as the RNA of virions, according to a recent study on poliovirus-infected HeLa cells (87). After the inhibition of host-cell RNA synthesis with actinomycin D, the RNA synthesized in the poliovirus-infected cell was analyzed by sucrose gradient sedimentation. Two virus-specific species were found: 35*S* and 16*S* RNA. The 35*S* RNA, corresponding to viral RNA, had base ratios close to those of viral RNA, as did the 16*S* RNA. Thus, an understanding of viral RNA replication depends on elucidation of the process whereby viral RNA of noncomplementary nature is the major product.

Development of Current Concepts of Viral RNA Synthesis

Early speculations (R. Dulbecco, *personal communication*) on the nature of viral RNA synthesis centered on two alternative hypothetical schemes (Fig. 1). In the first scheme, (a) host-cell DNA or modified host-cell DNA, or (b) some viral-induced DNA intermediate, would serve as a template for RNA synthesis. In the second scheme, (c) viral RNA would be synthesized directly from viral RNA. Since the structure of RNA was not known when these schemes were first discussed, it was not possible to specify the molecular details of these general alternatives. The inhibition of DNA synthesis, either before or after infection or both before and after infection with poliovirus, did not affect the yield of virus (71). Unfortunately, similar experiments with Newcastle disease virus (NDV) in HeLa cells are more difficult to interpret because of the very low yield of NDV in this cell system (71). But at least for poliovirus, newly synthesized DNA does not play a role in viral RNA synthesis. When cells were pretreated with 5-bromouracil to form highly abnormal DNA, and were subsequently infected with poliovirus of well-defined genotype, no increase in the mutation frequency of the progeny virus was noted (71). Thus, even pre-existing host-cell DNA does not play a role in RNA virus replication.

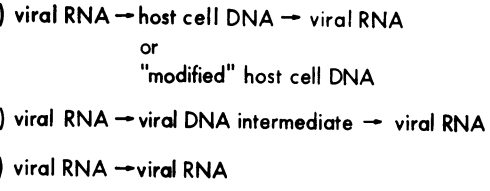

FIG. 1. *Early proposals concerning the mechanism of viral RNA biosynthesis.*

Mitomycin C induces breakdown (61, 69) and cross-linkage (41, 42, 76) of DNA. Whereas low doses inhibit only DNA synthesis, high doses inhibit both DNA synthesis and DNA-dependent RNA synthesis, the latter due to extensive destruction of DNA templates. Since even very high doses of mitomycin C did not affect

the yield of mengovirus, replication of this RNA virus does not require either associated or prior synthesis of DNA, or integrity or expression of any particular host-cell genome (57).

Actinomycin D inhibits DNA-dependent RNA synthesis (58, 59) by interaction with the DNA template (40, 60). Under conditions of total inhibition of cellular RNA synthesis by actinomycin D, there is a normal production of mengovirus (58, 59) and other viruses. It is also possible to demonstrate the synthesis of viral-specific RNA in these cells (26, 87). Thus, no host-cell genome need function during replication of some RNA viruses. Difficulties arise, however, when RNA virus synthesis *is* inhibited by actinomycin D (60). The basis of this inhibition is not yet clear but may be due to secondary effects of actinomycin in some cases. For example, inhibition of reovirus synthesis (34) is dependent on the concentration of actinomycin (68) and the length of time the cells are pretreated with actinomycin before infection (R. M. Franklin, *unpublished data*). A detailed discussion of RNA viruses sensitive to actinomycin is beyond the scope of this review.

A different approach to the problem of DNA involvement in RNA virus replication is the use of the homology test, in which denatured DNA forms a specific hybrid with homologous RNA. The failure to find such a hybrid when *Escherichia coli* DNA was tested with RNA from MS2 bacteriophage (18) indicates that DNA, either preexisting or newly synthesized, does not serve as a template for viral RNA synthesis. Thus, a variety of approaches to the problem have led to the conclusion that, for certain RNA viruses, (i) neither host-cell DNA nor any DNA synthesized after infection serves as template for viral RNA synthesis, and (ii) neither host-cell DNA nor any DNA synthesized after infection need express itself (by controlling the synthesis of specific RNA species) during viral RNA synthesis.

These principles have been demonstrated only with certain RNA viruses which have served as convenient model viruses. It is far from clear that the statement is valid for all RNA viruses. This qualification should be kept in mind throughout this entire review. At this time there is no evidence on the mechanism of replication of the double-stranded RNA of reovirus. In the case of the avian tumor viruses, both actinomycin and inhibitors of DNA synthesis block viral multiplication under certain conditions (6, 7, 77–80).

The data discussed above resulted, then, in consideration of the alternate possibility that viral RNA serves as template for further viral RNA synthesis.

Mengovirus-infected L cells have an altered pattern of RNA synthesis (27). There is a viral-induced inhibition of cellular (nuclear) RNA synthesis and an appearance of viral-specific (actinomycin-resistant) RNA synthesis in the cytoplasm (26, 27). These observations led to the search for an RNA polymerase responsible for synthesis of viral RNA. Evidence for such an enzyme was first demonstrated in the mengovirus-L cell system (10, 11), and further work with RNA phage-infected *E. coli* (35, 86) has fully confirmed the existence of such an enzyme (or enzymes).

An important study demonstrated the existence of a unique species of RNA in Krebs II ascites cells infected with encephalomyocarditis virus (EMC). This molecular species had properties of double-stranded RNA and it was infectious (54). By analogy with the double-stranded DNA found in ϕX174-infected bacteria, this double-stranded RNA was termed a *replicative form*. After this initial report, double-stranded RNA was detected in a number of different cells infected with RNA viruses, as will be discussed in more detail later. *Replicative form* may not be the best descriptive term for double-stranded RNA, since our present evidence indicates a more complicated structure to be the actual replicative form. Rather than add further confusion to the literature, we have decided to continue calling the double-stranded RNA *replicative form* and the more complex structure *replicative intermediate* (which will be discussed below).

In conclusion, one or several enzymes catalyze the polymerization of viral RNA from an RNA template. The precise nature of the template is not yet understood, but a double-stranded RNA or a more complex structure is involved.

BIOSYNTHESIS OF VIRAL RNA

Formation of Virus-Specific Double-Stranded RNA

For the purposes of this discussion, we will assume that the mechanisms of RNA replication for all small RNA-containing viruses are similar.

Montagnier and Sanders (54) first demonstrated conclusively that a replicative form of viral RNA appeared in infected cells. Two forms of virus-specific RNA were identified by sucrose gradient analysis of the RNA extracted from Krebs II ascites cells infected with EMC. One type of RNA had a sedimentation coefficient of 37*S*, the same as that of RNA extracted from intact virus. This RNA was infectious and, therefore, was the RNA eventually packaged into the virion. The second form of RNA had a sedimentation coefficient of 20*S* and was also infectious.

Unlike the 37*S* viral RNA or the host-cell RNA, this RNA was resistant to treatment with pancreatic ribonuclease. Its buoyant density in a Cs_2SO_4 equilibrium density gradient was 1.57 g/cc, while that of single-stranded EMC RNA was 1.63 g/cc. Upon heating, the 20*S* RNA displayed a temperature versus optical density profile similar to that exhibited by DNA, and a melting temperature of 96 C in 0.15 M NaCl plus 0.015 M sodium citrate. All of these characteristics suggested that the 20*S* component was a double-stranded form of viral RNA, analogous to the replicative form that had been described for the single-stranded DNA of ϕX174 (73). RNA with similar properties has also been found in poliovirus-infected HeLa cells by Baltimore, Becker, and Darnell (9). In this case, the replicative form was found in special cytoplasmic structures which were isolated from infected cells and which contained the viral RNA polymerase and most of the newly synthesized viral RNA. The replicative form of poliovirus RNA was infectious, just as was the replicative form of EMC virus RNA (55).

Interesting observations have also been made on RNA phage-infected *E. coli*. Growth of RNA phage is independent of DNA synthesis (14), and parental bacteriophage RNA is not transferred to progeny phage (15, 19). These facts must be taken into account when mechanisms of viral RNA replication are considered.

Parental RNA is converted to a ribonuclease-resistant molecule which has sedimentation properties consistent with those expected of a double-stranded RNA composed of a parental strand and its complement (21, 22, 45). Furthermore, chloramphenicol inhibited the conversion of parental RNA to the ribonuclease-resistant form. Therefore, an enzyme (or enzymes) not present in the uninfected cell is necessary for this conversion (21, 45).

Ribonuclease-resistant RNA can also be detected in bacteriophage-infected *E. coli* as a species of RNA which is labeled after a very short pulse of H^3-uridine (25). The time of appearance of this RNA, as well as the results of pulse-chase experiments, strongly suggests that it is a precursor of single-stranded phage RNA. The loss of ribonuclease-resistant label was approximately equal to the gain of 27*S* label during the chase. If the RNA were labeled for 15 min, a period long enough to label both strands of the RNA duplex, the ribonuclease-resistant label decreased during the subsequent chase to 25% of the initial quantity. These results suggest that, of the two strands in the ribonuclease-resistant core, one is turning over while the other may be stable.

These results supported the earlier experiments of Weissmann et al. (84, 85) which led to the proposal that viral RNA probably replicates by an asymmetrical semiconservative mechanism whereby newly formed, parental-like strands displace a parental-like strand from the duplex as synthesis proceeds. These experiments involved a hybridization technique whereby the total amount of replicative form in the infected cell could be accurately determined (84). Newly synthesized replicative form was detectable by 20 min after infection, although replicative form containing parental RNA was detectable by 5 or 6 min after infection. At 45 min after infection, approximately 1.7% of the total RNA in the cell consisted of replicative form; 20% of the total RNA was viral RNA.

Ribonuclease-resistant replicative forms of RNA have also been found in cells infected with several other animal and plant viruses. Among the plant viruses are turnip yellow mosaic virus (TYMV) (50, 56) and TMV (13, 70, 82).

Properties of Virus-Specific Double-Stranded RNA

Table 2 lists some characteristics of the replicative form of animal, plant, and bacterial RNA viruses. The assignment of a double-stranded structure to RF is justified by these and the following properties. (i) The molecule is resistant to hydrolysis by pancreatic ribonuclease, a known property of double-stranded RNA (28). Most of the preparations listed in Table 2, with the exception of two (1, 44), were prepared by ribonuclease treatment of an RNA preparation from infected cells. (ii) The double-helical structure of the replicative form of MS2 RNA has been directly demonstrated by X-ray diffraction analysis (47). (iii) Hybridization tests of denatured ribonuclease-resistant RNA with viral RNA indicate the presence of a strand of RNA complementary to viral RNA. Further, the nucleotide compositions of TMV RNA RF and of both components of the replicative form are compatible with a duplex structure containing a strand of viral RNA and a strand complementary to viral RNA (84, 82).

The values in Table 2 all reflect the double-helical structure of RF molecules. Although the reported buoyant densities vary considerably from laboratory to laboratory, all are lower than those of single-stranded RNA compared in a particular laboratory. The sedimentation rate of animal virus RF is greater than that of bacteriophage RF, a reflection of the higher molecular weight of animal virus RNA as compared with bacteriophage RNA.

TABLE 2. *Properties of replicative forms from various sources*

Virus	T_m			Buoyant density†		Sedimentation coefficient (*S*)	Reference
	Temp	Buffer	Measurement*	Cs_2SO_4	CsCl		
	C						
EMC	96	SSC‡	OD	1.57		20	54
EMC	84	1/10SSC	OD				54
Polio				1.65		16	9
Polio				1.58		16	55
MS2	103	SSC	Ribonuclease	1.609			83
MS2					1.868	13	44
fr	101	0.2 M NaCl	OD	1.609		14.2	43
M12	96	10^{-3} M NaCl	OD				1
R17			Ribonuclease	1.608		13.0	23
TMV	97	SSC		1.601		11.6	13

* Refers to type of assay for measuring T_m. OD = increase in absorbance at 260 mμ; ribonuclease = conversion of replicative form from a ribonuclease-resistant to a ribonuclease-sensitive form.

† Results expressed as grams per cubic centimeter.

‡ SSC = 0.15 M NaCl plus 0.015 M sodium citrate.

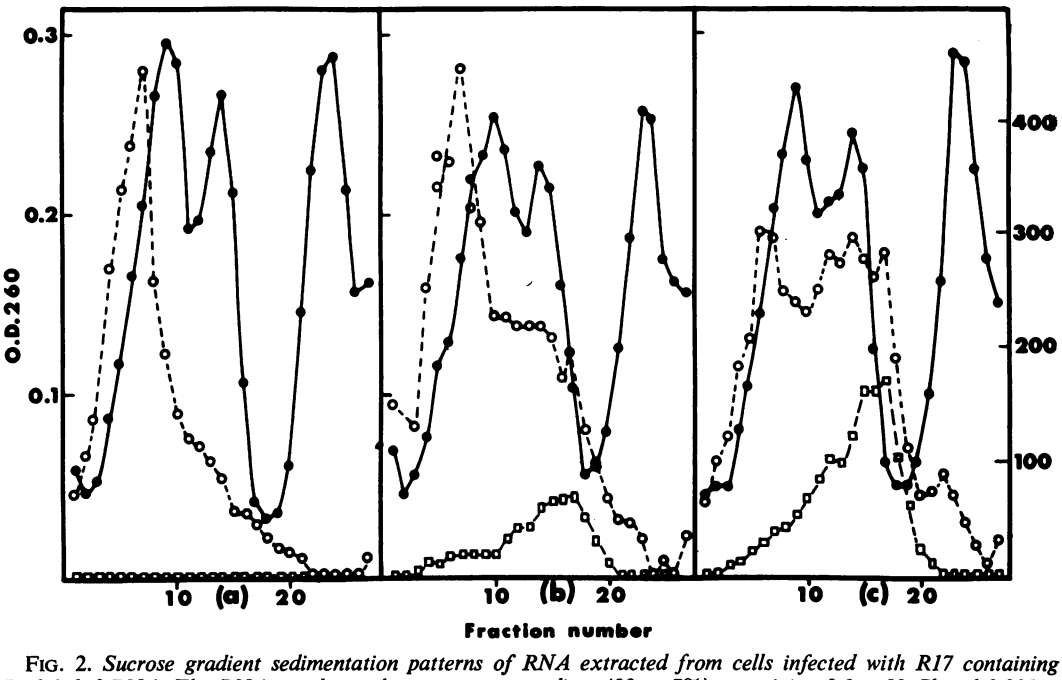

FIG. 2. *Sucrose gradient sedimentation patterns of RNA extracted from cells infected with R17 containing P^{32}-labeled RNA. The RNA was layered on a sucrose gradient (20 to 7%) containing 0.1 M NaCl and 0.001 M $MgCl_2$ and centrifuged at 37,500 rev/min for 4 hr at 12 C in a Spinco model L2 ultracentrifuge. Each fraction was diluted to 2 ml with 0.15 M NaCl, and the optical density (●) was determined at 260 mμ. A 1-ml amount was removed from each for treatment with ribonuclease (0.1 μg/ml, 10 min at 37 C). After precipitation with 10% trichloroacetic acid, the radioactivity for the untreated (○) and ribonuclease-treated (□) fractions was determined. Time after infection: (a) zero; (b) 6 min; (c) 12 min. This figure was originally published by Erikson et al. (21).*

Experiments have been performed in this laboratory (21, 25) to characterize all forms of viral-specific RNA in R17 bacteriophage-infected cells. The replicating molecule as isolated by phenol extraction at room temperature appears to be heterogeneous, as determined by sedimentation through a sucrose gradient. This is true for pulse-labeled molecules and also for molecules containing parental RNA. To observe this sedimentation heterogeneity, the assay for ribonuclease resistance must be carried out after centrifugation of the RNA (Fig. 2). If the RNA is

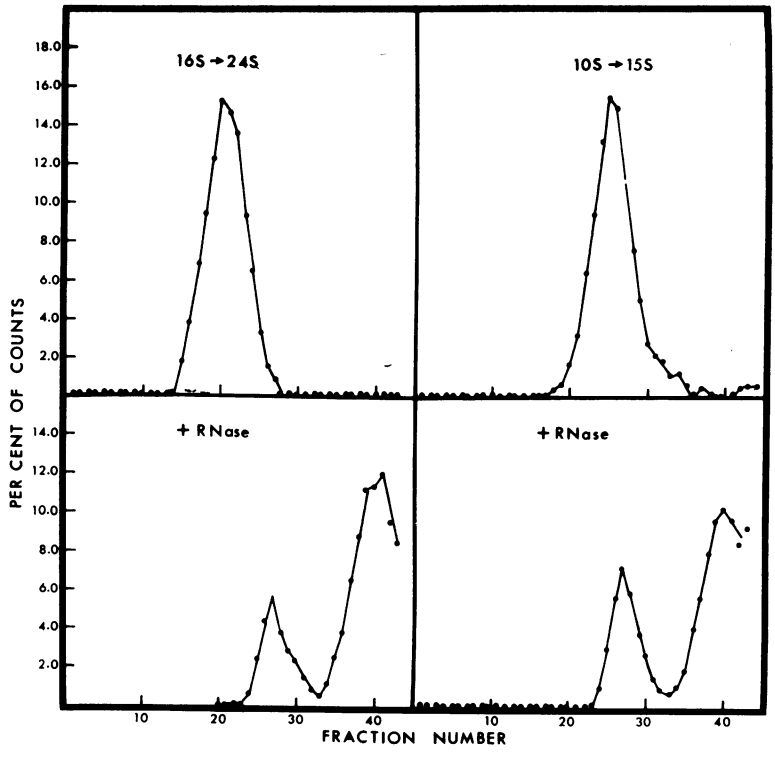

FIG. 3. *Analysis of the effect of pancreatic ribonuclease on sedimentation of the replicative intermediate. RNA was prepared and then centrifuged exactly as shown in Fig. 2. After fractionation, certain fractions were collected corresponding to 16S to 24S RNA and 10S to 15S RNA. These were then recentrifuged either with or without ribonuclease treatment (conditions described in the legend to Fig. 2). For simplicity, only the radioactivity of parental RNA is plotted.*

treated prior to centrifugation, a single homogeneous peak of resistant RNA is observed. As expected from this result, if RNA is first fractionated by centrifugation, the subsequent ribonuclease treatment of any fraction which contains ribonuclease-resistant RNA yields 13*S* ribonuclease-resistant molecules and nonsedimentable fragments (Fig. 3). These results led to the suggestion (25) that nascent strands of viral RNA are attached to a double-stranded form of RNA and the presence of these strands causes the complex to sediment more rapidly than the double-stranded core. This molecular species was designated (21) the *replicative intermediate* (RI) to distinguish it from the molecule previously described as the *replicative form* (54).

Examination of viral-specific RNA in other types of cells has revealed similar sedimentation patterns. For example, in actinomycin-treated chick embryo fibroblasts infected with Semliki forest virus, two viral RNA components were labeled with H^3-adenosine. These had sedimentation coefficients of 36*S* and 24*S* (74). Only the latter component was labeled during a short pulse, as with R17. Furthermore, ribonuclease treatment before sedimentation resulted in a shift of the *S* value of the labeled material to less than 24*S*, similar to the behavior of R17 replicative intermediate. Such properties have also been reported for foot-and-mouth disease virus (12).

Although not enough physicochemical data are available to establish definitively the structure of the complex which generates new strands of viral RNA, we consider that the structures shown in Fig. 4 are compatible with the data obtained to date. Double-stranded RNA (RF) is formed when parental RNA acts as a template for the synthesis of its complement. This step is probably controlled by a viral-specific polymerase. New viral strands are then synthesized on this template, perhaps in a direction opposite to that in which the complement was synthesized. New viral strands may be generated by a second enzyme, possibly a host-specific enzyme. The nascent strand is attached to the duplex template by hydrogen bonds close to the growing point of the chain. The population of duplexes with nascent strands would represent RI, and the different

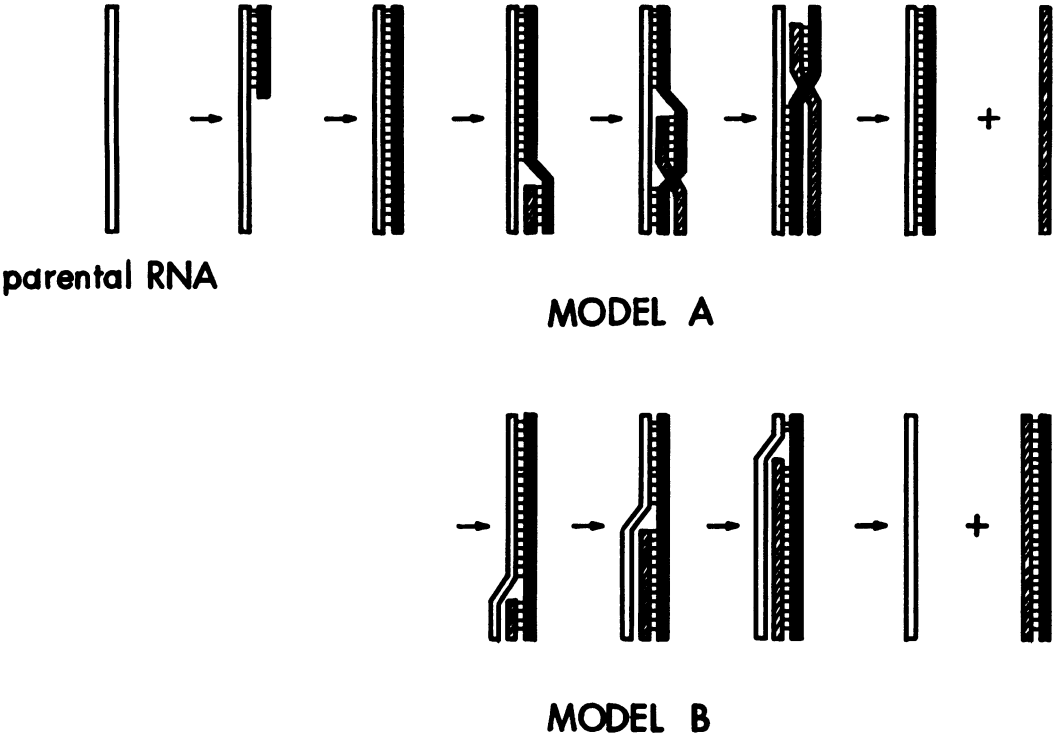

FIG. 4. *Mechanisms for the synthesis of viral RNA. In model A, the new viral RNA molecule would be generated on a stable duplex in a manner presumably analogous to the synthesis of RNA on a DNA duplex. In this case the new strand (cross-hatched bars) would be released, and the parental strand (clear bars) would remain in the duplex hydrogen-bonded to its complementary strand (solid bars). In model B (see 25), the viral strands would be displaced from the duplex during the synthesis of the new viral RNA strand which would remain hydrogen-bonded to the complementary strand. During the first round of synthesis in this case, the strand displaced would then be the parental strand originally in the duplex.*

lengths of the nascent chain would result in sedimentation heterogeneity of the molecules. There could be one or several nascent chains per duplex, although no information is available on this point. Completed viral chains would have a choice of three fates: to form new duplexes, to serve as messenger RNA, or to enter virions. This scheme (Fig 4, model A) is compatible with the fact that parental RNA is not transferred to progeny phage, because parental RNA would remain in the duplex. Alternatively, the viral RNA strand may be displaced from the complementary strand as a new strand is synthesized (Fig 4, model B). This scheme was suggested previously (25), but is not compatible with the lack of transfer of parental RNA to progeny phage (15, 19). There is a possibility that both mechanisms may be operative in one cell if a duplex containing the parental strand functions differently from other duplexes. Such behavior would be analogous to that of parental DNA-containing replicative form of ϕX174 (17).

The parental-labeled replicative intermediate RNA, which has a sedimentation coefficient of 12 to 16*S* (Fig. 2c), can be separated (22) into its component strands by thermal denaturation (Fig. 5). The 12 to 16*S* RNA, when resedimented without further treatment, displayed the expected sedimentation profile (Fig. 5a). However, when this RNA was heated for 3 min at 95 C to break hydrogen bonds and separate the strands, a new peak of parental-labeled RNA appeared upon subsequent sedimentation (Fig. 5b). This RNA had the same sedimentation rate as the R17 RNA added to the sample to serve as a sedimentation rate marker. Therefore, some parental RNA which had been converted to a double-stranded form after infection was then indistinguishable, by sedimentation analysis, from viral RNA. Moreover, after denaturation the RNA was no longer resistant to ribonuclease, an expected result of the separation of the strands.

In Fig. 5c and d, the ribonuclease-resistant 13*S* RNA (RF) is shown undenatured and de-

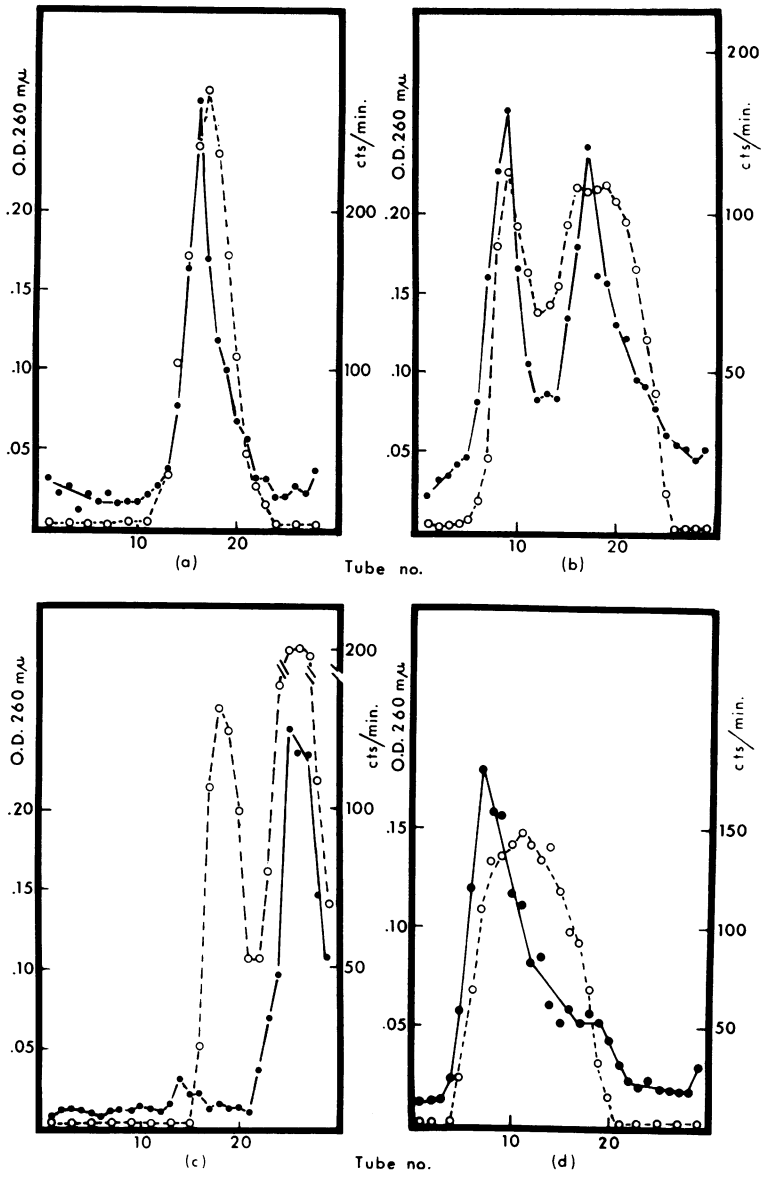

FIG. 5. *Analysis of 12 to 16S RNA extracted from bacteria infected for 12 min with P^{32}-labeled R17. (a) Sedimentation of an untreated sample. (b) Sedimentation of a sample denatured 3 min at 95 C in 10^{-3} M ethylenediaminetetraacetic acid (EDTA) plus 10^{-2} M tris(hydroxymethyl)aminomethane (Tris), pH 7.2, in the presence of 60 μg of unlabeled R17 RNA added as a sedimentation rate marker. (c) Sedimentation of a sample after ribonuclease treatment (0.1 μg/ml, 10 min, 37 C in 0.15 M NaCl). (d) Sedimentation of a sample of the ribonuclease-resistant RNA (Fig. 5c) after denaturation (3 min at 95 C in 10^{-3} M EDTA plus 10^{-2} M Tris, pH 7.2) in the presence of 60 μg of unlabeled R17 RNA. Each sample was centrifuged through a 4.4-ml sucrose gradient at 37,500 rev/min for 4 hr at 12 C. A 0.1-ml amount of each fraction was used for radioactivity determination (○) and the remainder was diluted for optical density determination (●). This figure was originally published by Erikson et al. (22).*

natured. The sedimentation profile after denaturation (Fig. 5d) indicates that the parental-labeled RNA is smaller and more heterogeneous than the parental RNA that entered the double-stranded form as well as that obtained from the sample untreated with ribonuclease (Fig. 5b). This suggests that ribonuclease hydrolyzes some bonds in the component strands of the double-

stranded molecule. Additional experiments are required to clarify the effects of ribonuclease on these molecules.

Viral-Induced RNA Polymerases

There are numerous reports concerning the enzymes responsible for the synthesis of the RNA which eventually appears in progeny virus, some of which will be mentioned here.

Virus-specific RNA polymerase activity was originally described by Baltimore and Franklin (10). This enzymatic activity was in the microsome fraction of mengovirus-infected L cells and catalyzed the incorporation of ribonucleotide triphosphates into RNA. The incorporation required Mg^{++} and was not inhibited by actinomycin D. Nearest neighbor analysis of the product revealed that the four triphosphates were incorporated in a nonrandom fashion. Using RNA polymerase from poliovirus-infected HeLa cells, Baltimore analyzed the product by sucrose gradient sedimentation (8). Radioactive label was incorporated into both 35*S* and 16*S* RNA by this system, and the 16*S* RNA had the properties expected of double-stranded RNA.

Induction of an analogous enzyme occurs in RNA phage-infected *E. coli* (86). A 50-fold purification did not free this particle-bound enzyme of RNA. All four triphosphates were required for maximal activity, but there was no requirement for added RNA. A similar enzmye was isolated from f2 bacteriophage-infected cells (4). Subsequently, the further purification of these enzymes has been carried out. The RNA synthetase from MS2-infected cells was purified in the form of haloenzyme, that is, in association with primer RNA. At least a part of the product of this enzyme was double-stranded RNA indistinguishable from the double-stranded RNA isolated from MS2-infected cells (84).

The f2-induced enzyme was purified over 100-fold. Unfortunately, this enzyme is somewhat unstable in the purified state, but is completely free from RNA and has a requirement for added primer RNA (5). Any single-stranded RNA can be utilized as primer. The base composition of the product synthesized is complementary to that of the RNA used to direct the reaction (67). The RNA product possesses a high degree of secondary structure as judged from the high T_m and the partial resistance to ribonuclease.

Very highly purified and specific RNA polymerases have been isolated from cells infected with the RNA bacteriophages Qβ or MS2 (35, 36, 75). Each polymerase is active only when homologous RNA is added to the reaction mixture. Identical copies of viral RNA are made in this system, since the amount of RNA synthesized is directly proportional to the increase in titer of infectious RNA. In addition, newly synthesized RNA is fully capable of serving as template for the polymerase. The presence of a replicative intermediate form of RNA which may have a function in the synthesis of the identical copies has not been reported, although sucrose gradient analysis of the RNA synthesized in this system suggests the presence of double-stranded molecules (37). Unfortunately, the ribonuclease sensitivity of this RNA has not been tested. The identification of RI should be easier in the cell-free system than in the intact cell where host RNA synthesis interferes with the analysis of virus-specific RNA (25).

Some amber mutants of bacteriophage f2 have an altered capacity to induce synthesis of RNA polymerase (48, 49). The experiments with these mutants suggest that in normal infection only input RNA strands are templates for polymerase synthesis, and progeny strands are templates for coat protein synthesis. However, in mutant-infected nonpermissive cells, some of the progeny RNA is used for synthesis of polymerase, and amounts in excess of that in wild-type infected cells are synthesized. At the same time, excess double-stranded RNA which has a sedimentation coefficient of about 7*S* is synthesized. Thus, the enzyme isolated from cells infected with this class of amber mutants probably converts parental RNA to a double-stranded form (67). An RNA-dependent RNA polymerase has also been isolated from Chinese cabbage leaves infected with TYMV RNA (2). The product of this reaction was partially resistant to ribonuclease and contained RNA complementary to the viral strand (3). This is further evidence for the generality of the system for synthesis of viral RNA.

CONCLUSIONS

A double-stranded or replicative form of viral RNA exists in a variety of cells infected with small viruses which contain one molecule of single-stranded RNA in the virion. This includes bacterial, plant, and animal cells infected under a wide variety of conditions. The exact role that double-stranded RNA plays in viral RNA synthesis is far from clear. Unfortunately, the evidence to date that it actually does play a role is only circumstantial. However, a normal Watson-Crick base-pairing mechanism for defining the sequence of the progeny virus RNA is highly attractive even in the absence of definitive experiments on the manner in which single strands are generated. Since double-stranded RNA is not produced if protein synthesis is inhibited at the

time of infection (21, 45), a viral-specific enzyme must be responsible for its production. In fact, there may be two enzymes (16) responsible for viral RNA synthesis: one which converts single strands to double-stranded molecules (67) and another which generates progeny RNA strands from these molecules.

Experiments in our laboratories are now directed towards an elucidation of the structure of the double-stranded molecule and of the role of the replicative intermediate in the synthesis of viral RNA.

ACKNOWLEDGMENT

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